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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*

It is hypothesized that the gene encoding RASGRF1, a GTP exchange factor (GEF), controls the severity of neurofibromatosis. **Over-expression of *Rasgrf1* is predicted to exacerbate neurofibromatosis while *Rasgrf1* silencing will attenuate it.** Two novel strains of mice ideally suited to test this hypothesis that were developed in my lab are being used to evaluate the role or *Rasgrf1* on the manisfestations of neurofibromatosis type 1. One strain of mice over-express *Rasgrf1*, the other has diminished expression. These were crossed with a mouse model for NF1 and the effects of the altered level of RASGRF1 on tumorigenesis were monitored. The results indicate that over-expression of *Rasgrf1* significantly hastens the time of tumor onset and increases the overall frequency of tumor incidence. In contrast, diminished expression modestly delays the timing of tumor development, but overall frequency of tumor development is not changed. To extend this work from the genetic to biochemical, we have established assays to evaluate Ras activation status. The analysis of tumors and tissues from mice is ongoing. To extend this work further from mouse to human, we are establishing the assays to detect human *RASGRF1* mRNA from human tumor sections using *in situ* hybridization.

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INTRODUCTION:

The goal of this research is to expand the knowledge of the genes that contribute to neurofibromatosis beyond the GAP1-related (GTPase activation protein-related) domain in the NF1 (neurofibromatosis 1) protein. It is hypothesized that the gene encoding RASGRF1, a GTP exchange factor (GEF), is one of these genes. **Over-expression of *Rasgrf1* is predicted to exacerbate neurofibromatosis while *Rasgrf1* silencing will attenuate it.** Two novel strains of mice ideally suited to test this hypothesis that were developed in my lab are being used to evaluate the role or *Rasgrf1* on the manisfestations of neurofibromatosis type 1.

BODY:

To test the influence or RASGRF1 on the manisfestations of neurofibromatosis type 1, we established crosses between a mouse model for NF1 and our mice that over- or under-express *Rasgrf1* (1) and Yoon et al. unpublished]. The NF1 model used is the so called "NP-cis" mice with lesions at *Nf1* and *p53* seven centimorgans apart on the same chromosome (2) Genotypic analysis of the progeny from this cross was done for *Nf1*, *p53* and the two separate alleles of *Rasgrf1*. A total of 123 animals were generated that included 75 with the original NP-cis genotype (NP), 25 mice with the NP-cis allele that also over-express *Rasgrf1* due to an activating mutation on the normally silent maternal allele (NP2) and 23 with the NP-cis allele that also under-express *Rasgrf1* due to an inactivating mutation on the single active paternal allele (NP3) and. The crosses were done in a manner that produced strain matched individuals so that the analysis of tumor incidence would not be confounded by strain background effects. The results indicate that over-expression of *Rasgrf1* in the NP2 animals significantly hastens the time of tumor onset and increases the overall frequency of tumor incidence. In contrast, diminished expression modestly delays the timing of tumor development, but overall frequency of tumor development is not changed (figure 1). These results demonstrate that *Rasgrf1* over-expression is a risk factor for tumorigenesis in the NP-cis mouse model of neurofibromatosis type 1.

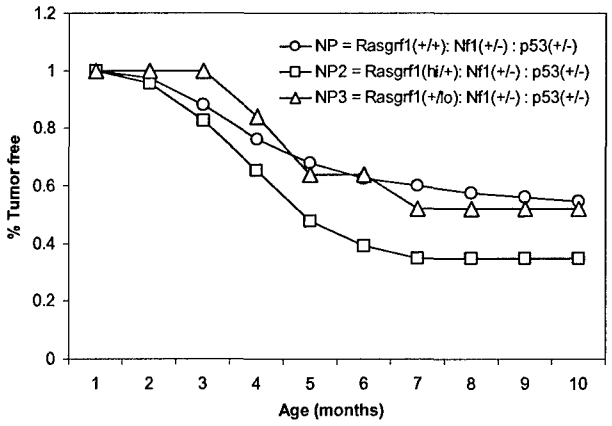


Figure 1. NP-cis mice (NP (2)) on the 129Sv background were bred with *Rasgrf1* mutant animals, also on the 129Sv background, with an inactivating lesion (Rasgrf1(lo/+), referred to as +/BJR3 below (1)) on the active paternal copy of this imprinted gene to produce NP3 animals, or the breeding was done with a transcription-activating mutation on the normally silent maternal allele (Rasgrf1(hi/+) referred to as BJR2/+ below, Yoon et al. unpublished) to produce NP2 mice. All animals were monitored for 10 months after birth for signs of tumor formation. Mice were sacrificed shortly after tumor onset and tissue removed for later histological analysis. $P<0.05$ by Mann Whitney testing.

Analysis of specific tumors isolated from the various animals did not reveal any readily apparent features suggesting that while there may be a quantitative difference in tumor incidence caused by altered expression of *Rasgrf1*, this did not lead to qualitative changes in the tumors that did arise.

We have also initiated the *in situ* hybridization experiments to evaluate *RASGRF1* presence in human tumors provided as paraffin sections on slides. The first studies are positive controls to validate the experimental techniques. These involve detecting the presence of RNA in mouse brain sections where *Rasgrf1* is known to be expressed. Signals have been obtained and optimization is under way to improve signal intensity.

An additional component of our work has been to evaluate in greater detail and at the molecular level the specific changes in gene expression and signaling events caused by the various *Rasgrf1* mutations we have generated. Specifically, we have done the work listed in the bullets below using mice harboring the various *Rasgrf1* alleles we have created and crossed with NF1 and NPcis mice. The detailed results follow the bullets.

- Quantify precisely the changes in *Rasgrf1* mRNA production and regions of production.

- Measure the amounts of *Rasgrf1* protein made and assay the extent of Ras activation

Assays for *Rasgrf1* mRNA To identify region in the brain expressing *Rasgrf1* and to determine if there are differences in *Rasgrf1* expression in the different regions of mouse brain, we performed PCR analysis using mRNA taken from the cerebrum, cerebellum and hippocampus. This assay was semiquantitative, using a five fold dilution series of cDNA prepared from these three sites. The results shown in figure 2 reveal that three are no large differences, by brain region, in *Rasgrf1* mRNA expression.

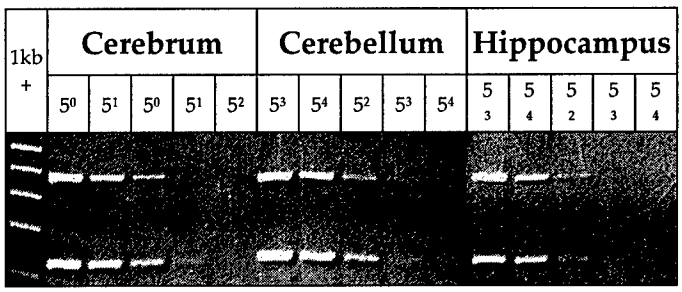


Figure 2. A dilution series of cDNAs prepared from mRNA isolated from different brain regions of wild type (+/+) mice was amplified by PCR and run on an agarose gel. Primers used detected *Rasgrf1*(top band) and *Rpl32*which was used as a loading control. No large differences were noted for the regions in which *Rasgrf1* is expressed, it appeared at comparable levels in each region tested.

To quantify more precisely the levels of *Rasgrf1* mRNA made by our various mutant mice, we performed real time PCR assays. mRNA was isolated from whole brains of mutant mice at different developmental stages, cDNAs prepared and real time PCR performed using TaqMan probes for fluorescence detection. The graphs in figure 3 show the results with the Y axis reporting the C_T values of the real time PCR which reflect the relative abundance of mRNAs from the mice. Note that high C_T values reflected low mRNA abundance and for every unit difference in C_T value, there is a two fold difference in mRNA abundance. BJR2/+ mice are the animals with biallelic expression of *Rasgrf1*, +/+ are wild type mice with paternal allele-specific expression and +/BJR3 mice lack expression from the single normally active allele in neonates. The data show that levels of *Rasgrf1* mRNA are approximately equal in BJR2/+ and +/+ neonates, despite the biallelic expression in the former while level in +/BJR3 are approximately 5% of that seen in the other two strains of mice. In adult brain, BJR2/+ mice had the highest level of *Rasgrf1*expression which was twice that seen in +/+ animals which was in turn twice that seen in +/BJR3 mice.

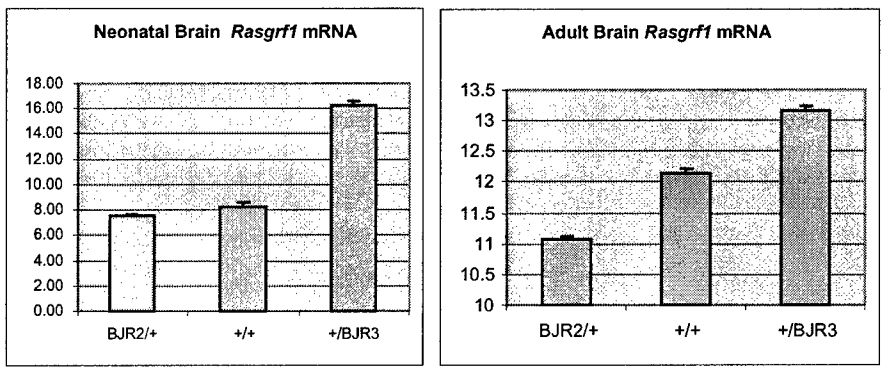
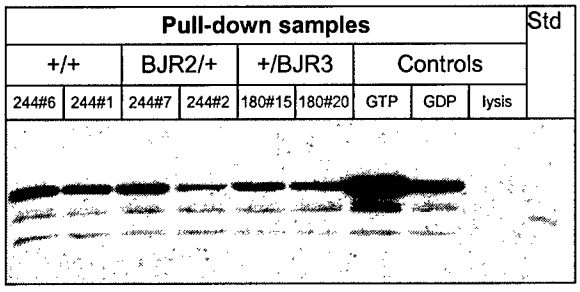


Figure 3. Real time PCR results for assays of *Rasgrf1* mRNA from mice.

Assay for Ras activation status and amount of Ras protein Because there are differences in the amount of expression in the brains of mice of the GTP exchange factor *Rasgrf1*, we wished to measure the extent of Ras activation as measured by the GTP-bound form of Ras. This was measured by a pull down assay using Raf-bound agarose beads – Raf preferentially binds Ras when it is in its GTP-bound state. The pulled down material was detected by Western blot and as a control for loading, the total amount of Ras in the input sample before pull down was measured as well. The results shown in figure 4 reveal no differences in Ras activation status or total Ras levels among +/+, BJR2/+ or +/BJR3 mice. This begs the question "what other signaling events may be modified by *Rasgrf1*expression that may cause the reduced survival in BJR2/+ mice shown in figure 1?"



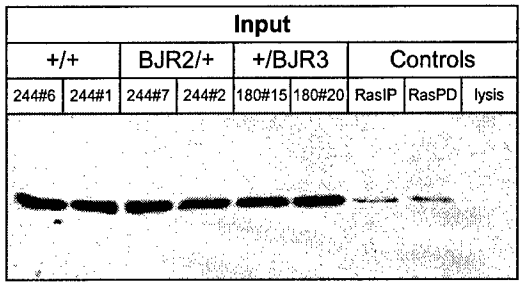


Figure 4. Ras activation status in +/+, BJR2/+ and +/BJR3 mice. Whole brain lysates from duplicate brains of mice were subjected to Raf-mediated pull down of Ras-GTP followed by Western blot analysis of Ras protein (left). The identifying numbers of each mouse are indicated. Controls included lysis buffer alone, or +/+ extracts incubated with GTP or GDP which demonstrated GTP incubation could enhance the activation status of Ras. Total lysates of each sample prior to pull down were also assayed by Western blot (right) for each mouse tested which revealed no significant differences among the mice. Controls included Ras protein that had been isolated by immune precipitation (IP) and also by Raf pull down (PD). Lysis buffer alone was included as a negative control.

KEY RESEARCH ACCOMPLISHMENTS:

- ☐ Development of the needed numbers and genotypes of mice needed to evaluate the role of RASGRF1 on tumor incidence in a neurofibromatosis type 1 model.
- ☐ Identification of over-expression of *Rasgrf1* as a contributing factor in tumor onset and frequency.
- ☐ Determination that loss of *Rasgrf1* expression produced no significant changes in tumor onset or frequency.
- ☐ Isolation of tumor tissues for histological analysis from NP-cis mice that are also manipulated for *Rasgrf1* expression.
- ☐ Determination that altered *Rasgrf1* expression produced no overt histological changes in tumors that arose.
- ☐ Identified three regions in the mouse brain expressing *Rasgrf1*.
- ☐ Quantified changes in *Rasgrf1* expression in mutant and wild type mice at different developmental stages by real time PCR.
- ☐ Completed assays for Ras activation status in brains of wild type and mutant mice.

REPORTABLE OUTCOMES:

Additional analysis of collected specimens is needed prior to reporting.

CONCLUSIONS:

This work demonstrates that in this animal model for neurofibromatosis type 1, over expression of *Rasgrf1* is a risk factor for faster tumor development and a higher frequency of tumor formation. If *RASGRF1* mRNA and protein are present in human tumors from NF1 patients, this suggests that therapeutics developed to antagonize the RASGRF1 protein may be of therapeutic benefit for them. Such antagonists have not been developed, however, their development may be justified by these studies. Molecular mechanisms by which *Rasgrf1* over expression may influence tumor development remain elusive. Our hypothesis that changes in Ras activation status are the key were not bourn out by our assays for GTP-bound Ras. Additional assays for other intracellular signaling events affected by *Rasgrf1* are warranted.

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